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CARBAMOYL-PHOSPHATE SYNTHETASE GENE OF CORYNEFORM BACTERIA AND METHOD FOR PRODUCING L-ARGININE

Cross-Reference to Related Application

This is a continuation-in-part of application Ser. No. 09/494,359 filed January 31, 2000.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to carbamoyl-phosphate synthetase of coryneform bacteria, and a gene therefore. The gene can be utilized for production of carbamoyl-phosphate synthetase and subunits thereof, breeding of L-arginine-producing bacteria and nucleic acid-producing bacteria and so forth.

Description of the Related Art

Carbamoyl-phosphate synthetase is an enzyme that catalyzes the reactions producing carbamoyl phosphate from carbonic acid, ATP and glutamine. Carbamoyl phosphate produced by these reactions serves as a source of carbamoyl group required for the reaction producing citrulline from ornithine in the L-arginine biosynthetic pathway. Furthermore, carbamoyl aspartate produced from aspartic acid and carbamoyl phosphate is one of the intermediates of the pyrimidine biosynthesis system including uridine 5'-monophosphate.

Carbamoyl-phosphate synthetase consists of two subunits, and it has been known for bacteria belonging to the genus *Escherichia* or *Bacillus* that those subunits are encoded by *carA* and *carB* genes.

However, as for coryneform bacteria, there have been no findings about the carbamoyl-phosphate synthetase activity and enzymes therefor, and any genes therefor have not been elucidated.

Incidentally, it has been reported that when a transformant of *Escherichia coli* to which introduced a plasmid harboring the genes *carA, carB, argI* and arg box was cultured in the medium added with glutamine which is substrate of carbamoyl-phosphate synthetase, the concentration of intracellular L-arginine was the same as that of a control strain to which only the vector was introduced. However, when the transformant was cultured in a medium added with glutamine accompanied with ornithine which is a substrate of ArgI together with carbamoyl phosphate, the concentration of intracellular L-arginine was higher than that of the control strain (Malamy M. et al., *Applied Environmental Microbiology*, 63(1), 33 (1997)). From these result, it was suggested that the rate-determining step of synthesis of L-arginine is supply of ornithine.

There was thought to be a possibility that the rate-determining step of supply of ornithine is N-acetylglutamine synthetase (ArgA). ArgA suffers feedback inhibition by the final product, L-arginine, in the biosynthesis pathway of *Escherichia coli*.

AS for the strain in which argA gene coding for feedback inhibition-desensitized ArgA was amplified by plasmid, the concentration of intracellular L-arginine was increased even in a medium added with only glutamine as well as in a medium added with both glutamine and ornithine. However, farther increase of concentration of intracellular L-arginine was not observed in the case that the strain was cultured with addition of glutamine, or glutamine and ornithin, also in the case that the both of *carA* and *carB* genes were further amplified in the strain (Malamy M. et al., *Applied Environmental Microbiology,* 64(5), 1805 (1998)).

On the other hand, any attempts have not been reported to enhance L-arginine productivity of microorganisms by utilizing a gene coding for carbamoyl-phosphate synthetase derived from coryneform bacterium.

SUMMARY OF THE INVENTION

An object of the present invention is to provide carbamoyl-phosphate synthetase of coryneform bacteria, a gene coding for it, and a method for producing L-arginine with a microorganism utilizing the gene.

The inventors of the present invention eagerly studied in order to achieve the aforementioned object. As a result, the inventors successfully obtained a DNA fragment containing the *carA* gene and the *carB* gene from a wild strain of *Brevibacterium lactofermentum by* utilizing a *carB*-deficient strain of *Escherichia coli*, and thus accomplished the present invention.

That is, the present invention provides the followings.

(1) A DNA fragment which encodes a polypeptide defined in the following (A) or (B):

(A) a polypeptide which has an amino acid sequence comprises at least the amino acid numbers 50 to 393 of the amino acid sequence of SEQ ID NO: 2,

(B) a polypeptide which has an amino acid sequence comprises at least the amino acid numbers 50 to 393 of the amino acid sequence of SED ID NO: 2 including substitution, deletion, insertion, addition, or inversion of one or several amino acids, and can constitute a protein having a carbamoyl-phosphate synthetase activity with a large subunit of carbamoyl-phosphate synthetase having an amino acid sequence comprising at least the amino acid numbers 55 to 1113 of SEQ ID NO: 3.

(2) A DNA fragment which encodes a polypeptide defined in the following (C) or (D):

(C) a polypeptide which has an amino acid sequence comprising at least the amino acid numbers 55 to 1113 of SEQ ID NO: 3,

(D) a polypeptide which has an amino acid sequence comprising at least the amino acid numbers 55 to 1113 of SEQ ID NO: 3 including substitution, deletion, insertion, addition, or inversion of one or several amino acids, and can constitute a protein having a carbamoyl-phosphate synthetase activity with a small subunit of carbamoyl-phosphate synthetase having an amino acid sequence comprises at least the amino acid numbers 50 to 393 of the amino acid sequence of SEQ ID No: 2.

(3) A DNA fragment encoding a polypeptide which has an amino acid sequence comprising at least the amino acid numbers 55 to 1113 of SEQ ID NO: 3 including substitution, deletion, insertion, addition, or inversion of one or several amino acids, and can constitute a protein having a carbamoyl-phosphate synthetase activity.

(4) A DNA fragment which encodes a polypeptide defined in the following (a) or (b), and a polypeptide defined in the following (c) or (d):

(a) a polypeptide which has an amino acid sequence comprising at least the amino acid numbers 50 to 393 in SEQ ID NO: 2,

(b) a polypeptide which has an amino acid sequence comprising at least the amino acid numbers 50 to 393 in SEQ ID NO: 2 including substitution, deletion, insertion, addition, or inversion of one or several amino acids, and can constitute a protein having a carbamoyl-phosphate synthetase activity with a large subunit of carbamoyl-phosphate synthetase having an amino acid sequence comprising at least the amino acid numbers 55 to 1113 of SEQ ID NO; 3,

(c) a polypeptide which has an amino acid sequence comprising at least the amino acid numbers 55 to 1113 of SEQ ID NO. 3,

(d) a polypeptide which has an amino acid sequence comprising at least the amino acid numbers 55 to 1113 of SEQ ID NO: 3 including substitution, deletion, insertion, addition, or inversion of one or several amino acids, and can constitute a protein having a carbamoyl-phosphate synthetase activity with a small subunit of carbamoyl-phosphate synthetase having an amino acid sequence comprising the amino acid numbers 50 to 393 in SEQ ID NO: 2.

(5) The DNA fragment according to (1), which has a nucleotide sequence comprising at least the nucleotide numbers 430 to 1461 in the nucleotide sequence of SEQ ID NO: 1.

(6) The DNA fragment according to (2), which has a nucleotide sequence comprising at least the nucleotide numbers 1756 to 4808 in the nucleotide sequence of SEQ ID NO: 1.

(7) The DNA fragment according to (3), which has a nucleotide sequence comprising at least the nucleotide numbers 430 to 4808 in the nucleotide sequence of SEQ ID NO: 1.

(8) A protein which comprises a polypeptide defined in the following (a) or (b), and a polypeptide defined in the following (c) or (d):

(a) a polypeptide which has an amino acid sequence comprising at least the amino acid numbers 50 to 393 in SEQ ID NO: 2,

(b) a polypeptide which has an amino acid sequence comprising at least the amino acid numbers 50 to 393 in SEQ ID NO: 2 including substitution, deletion, insertion, addition, or inversion of one or several amino acids, and can constitute a protein having a carbamoyl-phosphate synthetase activity with a large subunit of carbamoyl-phosphate synthetase having an amino acid sequence comprising at least the amino acid numbers 55 to 1113 of SEQ ID NO: 3,

(c) a polypeptide which has an amino acid sequence comprising at least the amino acid numbers 55 to 1113 of SEQ ID NO: 3,

(d) a polypeptide which has an amino acid sequence comprising at least the amino acid numbers 55 to 1113 of SEQ ID NO: 3 including substitution, deletion, insertion, addition, or inversion of one or several amino acids, and can constitute a protein having a carbamoyl-phosphate synthetase activity with a small subunit of carbamoyl-phosphate synthetase having an amino acid sequence comprising at least the amino acid numbers 50 to 393 in SEQ ID NO: 2.

(9) A coryneform bacterium which is transformed with a DNA fragment according to any one of (1) to (7).

(10) A microorganism which has enhanced intracellular carbamoyl-phosphate synthetase activity, and has L-arginine productivity.

(11) The microorganism according to (10) , wherein the enhanced intracellular carbamoyl-phosphate synthetase activity is obtained by increasing copy number of DNA encoding carbamoyl-phosphate synthetase of the microorganism, or by modifying an expression regulation sequence so that expression of the gene encoding carbamoyl-phosphate synthetase in the cell should be enhanced.

(12) The microorganism according to (11), wherein the DNA is a DNA fragment according to any one of (1) to (7).

(13) The microorganism according to (12), which is a coryneform bacterium.

(14) A method for producing of L-arginine, comprising the steps of culturing a coryneform bacterium according to any one of (10) to (13) in a medium to produce and accumulate L-arginine in the medium, and collecting the L-arginine from the medium.

The present invention provides genes coding for the subunits that constitute carbamoyl-phosphate synthetase. The gene can be utilized for production of carbamoyl-phosphate synthetase and subunits thereof, breeding of L-arginine producing bacteria and

nucleic acid-producing bacteria and so forth. Additionally, L-arginine can be produced efficiently according to the present invention.

BRIEF EXPLANATION OF THE DRAWINGS

Fig. 1 shows the structure of plasmid p19 containing the *carA* gene and *carB* gene.

Fig. 2 shows a construction process of plasmid pK1.

Fig. 3 shows a construction process of plasmid pSFK6.

DETAILED DESCRIPTION OF THE INVENTION

Hereafter, the present invention will be explained in detail.

<1> DNA of the present invention

The DNA of the present invention can be obtained from a chromosome DNA library of coryneform bacteria prepared with vectors such as plasmids by selection of the DNA using a microorganism which is deficient in *carA* or *carB*, for example, *Escherichia coli* RC50 (*carA*50, *tsx-273*, λ-, *rpsL135* (str^R), *malT1* (λR), *xylA7*, *thi-1*; Mol. Gen. Genet. ,133, 299 (1974)), *Escherichia coli* JEF8 (thr^-31, Δ*carB*, *relA*$^-$, *metBl*, Mol. Gen. Genet., 133, 299 (1974)) and so forth. Because a microorganism which is deficient in *carA* or *carB* exhibits L-arginine and uracil auxotrophy, a DNA fragment can be obtained by transforming such a microorganism with a chromosome DNA library, selecting clones in which the auxotrophy is complemented, and recovering a recombinant vector from the selected transformants.

The coryneform bacteria used for preparing a chromosome DNA library are not particularly limited, and examples thereof include bacteria having been hitherto classified into the genus *Brevibacterium* but united into the genus *Corynebacterium* at present (Ins. J. Syst. Bacteriol., 41, 255 (1981)), and include bacteria belonging to the genus *Brevibacterium*

closely relative to the genus *Corynebacterium*, more specifically, wild strains of *Brevibacterium lactofermentum* and so forth. Chromosome DNA of coryneform bacteria can be prepared by, for example, the method of Saito and Miura (Biochem. Biophys. Acta., 72, 619, (1963)), the method of K. S. Kirby (Biochem. J., 64, 405, (1956)) and so forth.

A chromosome DNA library can be obtained by partially digesting chromosome DNA with suitable restriction enzymes, ligating each of the obtained DNA fragments to a vector DNA autonomously replicable in *Escherichia coli* cells to prepare a recombinant DNA, and introducing the DNA into *Escherichia coli*. The vector is not particularly limited so long as it is a vector usually used for genetic cloning, and plasmid vectors such as pUC19, pUC18, pUC118, and pUC119, phage vectors such as λ phage DNA and so forth can be used. Further, a vector autonomously replicable in both of *Escherichia coli* cells and coryneform bacterium cells may also be used. Such a vector can be constructed by ligating a vector for *Escherichia coli* and pAM330, which is a cryptic plasmid of *Brevibacterium lactofermentum* (see Japanese Patent Laid-open No. 58-67699).

Specific examples of the vector autonomously replicable within both of *Escherichia coli* and coryneform bacterium cells include pSAC4 (see the examples mentioned below), pHK4 (see Japanese Patent Laid-open No. 5-7491) and so forth. *Escherichia coli* HB101 harboring pHK4 was designated as *Escherichia coli* AJ13136, and it was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry (postal code 305-8566, 1-3 Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan) on August 1, 1995, and received an accession number of FERM BP-5186.

The transformation of *Escherichia coli* cells can be performed by, for example, the method of D.A. Morrison (Methods in Enzymology, 68, 326, 1979), the method of treating

recipient cells with calcium chloride so as to increase the permeability of DNA (Mandel, M. and Higa, A., J. Mol. Biol., 53, 159 (1970)) and so forth. As for methods for preparation of chromosome DNA library, preparation of plasmid DNA, and digestion and ligation of DNA, as well as methods for PCR, preparation of oligonucleotides and hybridization mentioned hereinafter, conventional methods well known to those skilled in the art can be used. Such methods are described in Sambrook, J., Fritsch, E.F. and Maniatis, T., "Molecular Cloning, A Laboratory Manual, Second Edition", Cold Spring Harbor Laboratory Press, (1989) and so forth.

A nucleotide sequence of a DNA fragment containing *carA* and *carB* obtained as described above is represented as SEQ ID NO: 1 in the Sequence Listing. This sequence contains two open reading frames (ORF, nucleotide numbers 283 to 1461 and nucleotide numbers 1756 to 4808). The upstream ORF is *carA*, and the downstream ORF is *carB*. The amino acid sequences encoded by these ORFs are shown in SEQ ID NOS: 2 and 3, respectively. According to the present invention, a peptide encoded by *carA* is referred to as a small subunit, and a peptide encoded by *carB* is referred to as a large subunit.

As for the coding region of *carA*, GTG of the nucleotide numbers 283 to 285 is indicated as the initiation codon in Sequence Listing. However, GTG of the nucleotide numbers 415 to 417 or ATG of the nucleotide numbers 430 to 432 may possibly be the initiation codon. In any case, an active small subunit can be obtained by using a longer open reading frame for the upstream region for the expression of *carA*. Similarly, as for the coding region of *carB*, ATG of the nucleotide numbers 1470 to 1472 is indicated as the initiation codon in the Sequence Listing. However, GTG of the nucleotide numbers 1575 to 1577 or ATG of the nucleotide numbers 1632 to 1634 may possibly be the initiation codon. In any case, an active large subunit can be obtained by using a longer open reading frame for the upstream region for the expression of *carB*.

The amino acid corresponding to the GTG which is a possible initiation codon is indicated as valine for each subunit, but it may be methionine, valine or formylmethionine.

The small subunit of the carbamoyl-phosphate synthetase of the present invention is, for example, a polypeptide having the amino acid sequence of the amino acid numbers 50 to 393 in SEQ ID NO: 2, polypeptide having the amino acid sequence of the amino acid numbers 45 to 393 in SEQ ID NO: 2, polypeptide having the amino acid sequence of the amino acid numbers 1 to 393 in SEQ ID NO: 2 or the like.

The large subunit of the carbamoyl-phosphate synthetase of the present invention is, for example, a polypeptide having the amino acid sequence of the amino acid numbers 55 to 1113 in SEQ ID NO: 3, a polypeptide having the amino acid sequence of the amino acid numbers 36 to 1113 in SEQ ID NO: 3, a polypeptide having the amino acid sequence of the amino acid numbers 1 to 1113 in SEQ ID NO: 3, or the like.

According to the present invention, the DNA coding for the small subunit may be one coding for an amino acid sequence comprising at least the amino acid numbers 50 to 393 in SEQ ID NO: 2 including substitution, deletion, insertion, addition, or inversion of one or several amino acids, or one coding for a polypeptide which can constitute a protein having a carbamoyl-phosphate synthetase activity with the large subunit.

According to the present invention, the DNA coding for the large subunit may be one coding for an amino acid sequence comprising at least the amino acid numbers 55 to 1113 in SEQ ID NO: 3 including substitution, deletion, insertion, addition, or inversion of one or several amino acids, or one coding for a polypeptide which can constitute a protein having a carbamoyl-phosphate synthetase activity with the small subunit. Alternatively, it may be one coding for a protein which has the amino acid sequence comprising at least the amino acid numbers 55 to 1113 in SEQ ID NO: 3 including substitution, deletion, insertion, addition, or inversion of one or several amino acids, and has a carbamoyl-phosphate synthetase activity.

Furthermore, a DNA that encodes carbamoyl-phosphate synthetase containing a mutation or mutations in the small subunit or the large subunit, or both of them, also falls within the scope of the DNA of the present invention.

The term "several amino acids" preferably means 1 to 20 amino acids, more preferably 1 to 10 amino acids.

DNA, which encodes the substantially same peptide as the small subunit or the large subunit as described above, is obtained, for example, by modifying the nucleotide sequence of the DNA encoding the small subunit or the large subunit, for example, by means of the site-directed mutagenesis method so that one or more amino acid residues at a specified site of the gene involve substitution, deletion, insertion, addition, or inversion. DNA modified as described above may be obtained by the conventionally known mutation treatment. The mutation treatment includes a method for treating DNA coding for the small subunit or the large subunit *in vitro*, for example, with hydroxylamine, and a method for treating a microorganism, for example, a bacterium belonging to the genus *Escherichia* harboring DNA coding for the small subunit and the large subunit with ultraviolet irradiation or a mutating agent such as N-methyl-N'-nitro-N-nitrosoguanidine (NTG) and nitrous acid usually used for the mutation treatment.

The substitution, deletion, insertion, addition, or inversion of nucleotide as described above also includes mutation (mutant or variant) which naturally occurs, for example, the difference in strains, species or genera of the microorganism having the small subunit and/or the large subunit.

The DNA, which encodes substantially the same protein as carbamoyl-phosphate synthetase, is obtained by expressing DNA having mutation as described above in an appropriate cell, and investigating the carbamoyl-phosphate synthetase activity of an

expressed product. The carbamoyl-phosphate synthetase activity can be measured by the known method (Journal of General Microbiology, 136, 1177-1183 (1990)). The DNA, which encodes substantially the same protein as carbamoyl-phosphate synthetase, is also obtained by isolating DNA which is hybridizable with DNA having, for example, a nucleotide sequence corresponding to nucleotide numbers of 283 to 1461 or 1756 to 4808 of the nucleotide sequence of SEQ ID NO: 2, under stringent condition, and which encodes a protein having the carbamoyl-phosphate synthetase activity, from DNA coding for carbamoyl-phosphate synthetase having mutation or from a cell harboring it. The "stringent condition" referred to herein is a condition under which so-called specific hybrid is formed, and non-specific hybrid is not formed. It is difficult to clearly express this condition by using any numerical value. However, for example, the stringent condition includes a condition under which DNA's having high homology, for example, DNA's having homology of not less than 70%, preferably not less than 80%, more preferably not less than 90% are hybridized with each other, and DNA's having homology lower than the above are not hybridized with each other. Alternatively, the stringent condition is exemplified by a condition under which DNA's are hybridized with each other at a salt concentration corresponding to an ordinary condition of washing in Southern hybridization, i.e., 60°C, 1 x SSC, 0.1% SDS, preferably 0.1 x SSC, 0.1% SDS.

As a probe, a partial sequence of the nucleotide sequence of SEQ ID NO: 1 can also be used. Such a probe may be prepared by PCR using oligonucleotides produced based on the nucleotide sequence of SEQ ID NO: 1 as primers, and a DNA fragment containing the nucleotide sequence of SEQ ID NO: 1 as a template. When a DNA fragment in a length of about 300 bp is used as the probe, the conditions of washing for the hybridization consist of, for example, 50°C, 2 x SSC, and 0.1% SDS.

Because the nucleotide sequence of the DNA of the present invention has been elucidated, the DNA of the present invention can be obtained by amplifying it from coryneform bacterial chromosome DNA through polymerase chain reaction (PCR: polymerase chain reaction; see White, T.J. et al., Trends Genet., 5, 185 (1989)) utilizing oligonucleotides prepared based on that nucleotide sequence as primers, or by selecting it from a coryneform bacterial chromosome DNA library by hybridization utilizing an oligonucleotide prepared based on that nucleotide sequence as a probe. As nucleotide sequences of the primers used for PCR, a region upstream from the nucleotide number 283, preferably a region upstream from the nucleotide number 185 of SEQ ID NO: 1 can suitably be selected as the 5' primer, and a region downstream from the nucleotide number 4808 of SEQ ID NO: 1 can suitably be selected as the 3' primer.

Examples of the host for the expression of the DNA of the present invention include various bacteria such as *Escherichia coli* and coryneform bacteria including *Brevibacterium lactofermentum* and *Brevibacterium flavum*, eukaryotic cells such as those of *Saccharomyces cerevisiae* and so forth. In order to introduce the DNA of the present invention into these hosts, the host cells can be transformed with a recombinant vector obtained by inserting the DNA of the present invention into a vector selected according to the nature of the host in which the DNA is to be expressed. This procedure can be performed by a method well known to those skilled in the art. Specific examples of the method include the methods used for transformation of *Escherichia coli* mentioned above, the method in which competent cells are prepared from cells at the proliferating stage to introduce DNA, as reported for *Bacillus subtilis* (Duncan, C.H., Wilson, G.A. and Young, F.E., Gene, 1, 153 (1977)), the method in which DNA recipient cells are allowed to be in a state of protoplasts or spheroplasts capable of incorporating recombinant DNA with ease to introduce recombinant DNA into the DNA recipient cells, as known for *Bacillus subtilis,* actinomycetes, and yeasts (Chang, S. and

Choen, S.N., Molec. Gen. Genet., 168, 111 (1979); Bibb, M.J., Ward, J.M. and Hopwood, O.A., Nature, 274, 398 (1978); Hinnen, A., Hicks, J.B. and Fink, G.R., Proc. Natl. Acad. Sci. USA, 75, 1929 (1978)), the electric pulse method useful for coryneform bacteria (refer to Japanese Patent Publication Laid-Open No. 2-207791) and so forth.

The DNA to be introduced into the host such as those mentioned above may be DNA containing either *carA* or *carB*, or DNA containing both of them. Further, in order to attain efficient expression of these genes, a promoter functioning in the host cells such as *lac, trp* and P_L may be ligated at a position upstream from *carA* or *carB*.

Carbamoyl-phosphate synthetase or its subunits can be produced by culturing a transformant such as those mentioned above under a condition that allows the expression of *carA* or *carB*. The DNA of the present invention can also be utilized for breeding of L-arginine-producing bacteria or nucleic acid-producing bacteria such as uracil-producing bacteria. That is, a transformant introduced with the DNA of the present invention, in particular, one introduced with either *carA* or *carB* or both of them, should have increased carbamoyl-phosphate synthetase activity compared with non-transformants. Consequently, its productivity for L-arginine or nucleic acid such as uracil is improved.

<2> Method for producing L-arginine according to the present invention

L-Arginine can efficiently be produced by culturing a microorganism that has enhanced intracellular carbamoyl-phosphate synthetase activity, and has L-arginine productivity in a medium to produce and accumulate L-arginine in the medium, and collecting the L-arginine from the medium.

Specific examples of the microorganism having L-arginine productivity include coryneform bacteria, bacteria belonging to the genera *Bacillus*, *Serratia* and *Escherichia*,

yeast species belonging to the genus *Saccharomyces* or *Candida*. Of these, coryneform bacteria are preferred.

Exemplary specific species include *Bacillus subtilis* as a bacterium belonging to the genus *Bacillus*, *Serratia marcescens* as a bacterium belonging to the genus *Serratia*, *Escherichia coli* as a bacterium belonging to the genus *Escherichia*, *Saccharomyces cerevisiae* as a yeast species belonging to the genus *Saccharomyces*, *Candida tropicalis* as a yeast species belonging to the genus *Candida* and so forth.

Exemplary microorganisms having L-arginine productivity include *Bacillus subtilis* resistant to 5-azauracil, 6-azauracil, 2-thiouracil, 5-fluorouracil, 5-bromouracil, 5-azacytosine and so forth, *Bacillus subtilis* is resistant to arginine hydroxamate and 2-thiouracil, Bacillus subtilis resistant to arginine hydroxamate and 6-azauracil (see Japanese Patent Laid. open No. 49-1268191),

Bacillus subtilis resistant to histidine analogies or tryptophan analogues (see Japanese Patent Laid-open No. 52-114092),

a mutant of *Bacillus subtilis* exhibiting auxotrophy for at least one of methionine, histidine, threonine, praline, isoleucine' lysine, adenine, guanine and uracil (or uracil precursor) (see Japanese Patent Laid-open NO. 52-99289),

Bacillus subtilis resistant to arginine hydroxamate (see Japanese Patent Publication No. 51-6754),

Serratia marcescens exhibiting succinic acid auxotrophy or resistance to nucleic acid base analogies (Japanese Patent Laid-open No. 58-9692),

Serratia marcescens deficient in ability to metabolize arginine and exhibiting resistance to arginine antagonists and canavanine and auxotorophy for lysine (see Japanese Patent Laid-open No. 52-8729),

Escherichia coli introduced with the *argA* gene (see Japanese Patent Laid-open No. 57-5693),

Saccharomyces cerevisiae resistant to arginine, arginine hydroxamate, homoarginine, D-arginine and canavanine, or resistant to arginine hydroxamate and 6-azauracil (see Japanese Patent Laid-open No. 53-143288),

Candida tropicalis resistant to canavanine (see Japanese Patent Laid-open No. 53-3586) and so forth.

Coryneform bacteria include those bacteria having been hitherto classified into the genus *Brevibacterium* but united into the genus *Corynebacterium* at present (Ins. J. Syst. Bacteriol., 41, 255 (1981)), and include bacteria belonging to the genus *Brevibacterium* closely relative to the genus *Corynebacterium*. Examples of such coryneform bacteria are listed below.

Corynebacterium acetoacidophilum

Corynebacterium acetoglutamicum

Corynebacterium alkanolyticum

Corynebacterium callunae

Corynebacterium glutamicum

Corynebacterium lilium (Corynebacterium glutamicum)

Corynebacterium melassecola

Corynebacterium thermoaminogenes

Corynebacterium herculis

Brevibacterium divaricatum

(Corynebacterium glutamicum)

Brevibacterium flavum

(Corynebacterium glutamicum)

Brevibacterium immariophilum

Brevibacterium lactofermentum

(Corynebacterium glutamicum)

Brevibacterium roseum

Brevibacterim saccharolyticum

Brevibacterium thiogenitalis

Brevibacterium album

Brevibacterium cerinum

Microbacterium ammoniaphilum

The corynefonm bacteria that have the L-arginine productivity are not particularly limited so long as they have the L-arginine productivity. They include, for example, wild-type strains of coryneform bacteria; coryneform bacteria resistant to certain agents including sulfa drugs, 2-thiazolealanine, α-amino-β-hydroxyvaleric acid and the like; coryneform bacteria exhibiting L-histidine, L-proline, L-threonine, Lisoleucine, L-methionine, or L-tryptophan auxotrophy in addition to the resistance to 2-thiazolealanine (Japanese Patent Laid-open No. 54-44096); coryneform bacteria resistant to ketomalonic acid, fluoromalonic acid, of monofluoroacetic acid (Japanese Patent Laid-open No. 57-18989); coryneform bacteria resistant to argininol (Japanese Patent Laid-open No. 62-24075); coryneform bacteria resistant to X-guanidine (X represents a derivative of fatty acid or aliphatic chain, Japanese Patent Laid-open No. 2-186995) and so forth. Specifically, the following bacterial strains can be exemplified.

Brevibacterium flavum AJ11169 (FERM BP-6892)

Brevibacterium lactofermentum AJ12092 (FERM BP-6906)

Brevibacterium flavum AJ11336 (FERM BP-6893)

Brevibacterium flavum AJ11345 (FERM BP-6893)

Brevibacterium lactofermentum AJ12430 (FERM BP-2228)

The AJ11169 strain and the AJ12092 strain are the 2-thiazolealanine resistant strains mentioned in Japanese Patent Laid-open No. 54-44096, the AJ11336 strain is the strain having argininol resistance and sulfadiazine resistance mentioned in Japanese Patent Publication No. 62-24075, the AJ11345 strain is the strain having argininol resistance, 2-thiazolealanine resistance, sulfaguanidine resistance, and exhibiting histidine auxotrophy mentioned in Japanese Patent Publication No. 62-24075, and the AJ12430 strain is the strain having octylguanidine resistance and 2-thiazolealanine resistance mentioned in Japanese Patent Laid-open No. 2-186995.

The intracellular carbamoyl-phosphate synthetase activity of such microorganisms having the L-arginine productivity as mentioned above can be enhanced by, for example, increasing copy number of a gene coding for the carbamoyl-phosphate synthetase in the cells of the aforementioned microorganisms. The enhancement of the carbamoyl-phosphate synthetase activity can also be achieved by, in addition to the aforementioned gene amplification, modifying an expression regulation sequence for the DNA coding for carbamoyl-phosphate synthetase so that expression of the DNA gene coding for carbamoyl-phosphate synthetase should be enhanced. Specifically, an expression regulation sequence such as a promoter for a gene coding for carbamoyl-phosphate synthetase on the chromosomal DNA or a plasmid can be replaced with a stronger one (see Japanese Patent

Laid-open No. 1-215280). Strong promoters, which function in cells of coryneform bacteria, include *lac* promoter, *tac* promoter, *trp* promoter, of *Escherichia coli* (Y. Morinaga, M. Tsuchiya, R. Miwa and K. Sano, J. *Biotech., 5,* 305-312 (1987)) and the like. In addition, *trp* promoter of *Corynebacterium* bacteria is also a preferable promoter (Japanese Patent Laid-open No. 62-195294). By the replacement with these promoters the carbamoyl-phosphate synthetase activity is enhanced. The modification of expression regulation sequence may be combined with the increasing of the copy number of DNA coding for carbamoyl-phosphate synthetase. Further, the intracellular carbamoyl-phosphate synthetase activity can be enhanced by introducing one or more mutations into the enzyme protein of carbamoyl-phosphate synthetase so that the specific activity of the enzyme should be increased.

Examples of the DNA coding for carbamoyl-phosphate synthetase include the aforementioned *carA* and *carB* genes of *Brevibacterium lactofermentum* and one containing both of them.

Examples of the vector for introducing DNA coding for carbamoyl-phosphate synthetase into a microorganism include vectors autonomously replicable in cells of the microorganism. Specifically, the aforementioned vectors autonomously replicable in *Escherichia coli* cells, and the vectors autonomously replicable in both of *Escherichia coli* cells and coryneform bacterium cells.

The medium used for culturing a microorganism having enhanced intracellular carbamoyl-phosphate synthetase activity and L-arginine productivity obtained as described above may be a well-known medium conventionally used for the production of amino acids by fermentation. That is, it is a usual medium that contains a carbon source, nitrogen source, inorganic ions, and other organic components as required.

As the carbon source, it is possible to use sugars such as glucose, sucrose, lactose,

galactose, fructose and starch hydrolysates; alcohols such as glycerol and sorbitol; or organic acids such as fumaric acid, citric acid and succinic acid and so forth.

As the nitrogen source, it is possible to use inorganic ammonium salts such as ammonium sulfate, ammonium chloride and ammonium phosphate, organic nitrogen such as soybean hydrolysates, ammonia gas, aqueous ammonia and so forth.

The medium preferably contains a suitable amount of required substance such as vitamin B_1 and L-homoserine, yeast extract and so forth as trace amount organic nutrients. Other than those substances, a small amount of potassium phosphate, magnesium sulfate, iron ions, manganese ions and so forth may be added to the medium.

The cultivation is preferably performed under an aerobic condition for 1-7 days. Cultivation temperature is preferably 24-37°C, and pH of the medium during the cultivation is preferably 5-9. Inorganic or organic acidic or alkaline substances, ammonia gas and so forth may be used for adjusting pH. L-Arginine can usually be recovered from the fermentation medium by a combination of known techniques such as ion exchange resin method.

Best Mode for Carrying out the Invention

Hereafter, the present invention will be explained more specifically with reference to the following examples.

Example 1: Cloning of *carA* and *carB* of *Brevibacterium lactofermentum*

<1> Preparation of chromosome DNA of *Brevibacterium lactofermentum* ATCC13869

Brevibacterium lactofermentum ATCC13869 *was* inoculated to 100 ml of T-Y culture medium (1% of BactoTrypton Difco), 0.5% of Bacto-Yeast Extract (Difco) 0.5% of NaCl

(pH 7.2)), and cultured at a temperature of 31.5°C for 8 hours to obtain a culture. The culture was centrifuged at 3,000 r.p.m. for 15 minutes to obtain 0.5 g of wet bacterial cells, and chromosome DNA was obtained from the bacterial cells according to the method of Saito and Miura *(Biochem. Biophys. Acta ., 72,* 619 (1963)). Then, 60 μg of the chromosome DNA and 3 units of restriction enzyme *Sau*3AI were each mixed in 10 mM Tris-HCl buffer (containing 50 mM NaCl, 10 mM $MgSO_4$ and 1 mM dithiothreitol (pH 7.4)), and allowed to react at a temperature of 37°C for 30 minutes. The reaction mixture was subjected to phenol extraction and ethanol precipitation in a conventional manner to obtain 50 μg of chromosome DNA fragments of *Brevibacterium lactofermentum* ATCC13869 digested with *Sau*3AI.

<2> Preparation of gene library of *Brevibacterium lactofermentum* ATCC13869 using plasmid vector DNA

As a plasmid vector DNA autonomously replicable in both of *Escherichia coli* cells and coryneform bacterium cells, pSAC4 was used. pSAC4 was prepared as follows. In order to make a vector pHSG399 for *Escherichia coli* (Takara Shuzo) autonomously replicable in coryneform bacterium cells, a replication origin of the previously obtained plasmid pHM1519 autonomously replicable in coryneform bacterium cells (Miwa, K. et al., *Agric. Biol. Chem., 48 (1984)* 2901-2903) was introduced into the vector (Japanese Patent Laid-open No. 5-7491). Specifically, pHM1519 was digested with restriction enzymes *Bam*HI and *Kpn*I to obtain a gene fragment containing the replication origin, and the obtained fragment was blunt-ended by using Blunting Lit produced by Takara Shuzo, and inserted into the *Sal*I site of pHSG399 using a *Sal*I linker (produced by Takara Shuzo) to obtain pSAC4.

In 50 mM Tris-HCl buffer (containing 100 mM NaCl and 10 mM magnesium sulfate (pH 7.4)), 20 μg of pSAC4 and 200 units of a restriction enzyme *Bam*HI were mixed, and

allowed to react at a temperature of 37°C for 2 hours to obtain a digestion solution. This solution was subjected to phenol extraction and ethanol precipitation in a conventional manner. Then, in order to inhibit re-ligation of the DNA fragments derived from the plasmid vector, the DNA fragments were dephosphorylated with bacterial alkaline phosphatase according to the method described in Molecular Cloning, 2nd Edition (J. Sambrook, E.F. Fritsch and T. Maniatis, Cold Spring Harbor Laboratory Press, p1.56 (1989)), and subjected to phenol extraction and ethanol precipitation in a conventional manner.

To 66 mM Tris-HCl buffer {pH 7.5) containing 66 mM magnesium chloride, 10 mM dithiothreitol and 10 mM ATP, 1 μg of the pSAC4 digested with *Bam*HI, 1 μg of the chromosome DNA fragments of *Brevibacterium lactofermentum* ATCC13869 digested with *Sau*3AI obtained in Example l, and 2 units of T4 DNA ligase (produced by Takara Shuzo) were added, and allowed to react at a temperature of 16°C for 16 hours to ligate the DNA. Then, *Escherichia coli* DH5 was transformed with this DNA mixture in a conventional manner, and plated on an L agar medium containing 170 μg/ml of chloramphenicol to obtain about 20,000 colonies, which were used as a gene library.

<3> Transformation of *carB*-deficient strain of *Escherichia coli* (JEF8)

The *carB*-deficient strain of *Escherichia coli,* JEF8 (thr$^-$31, Δ*carB*, *relA*$^-$, *metBl*; *Mol. Gen. Genet.*, 133, 299 (1974)) was transformed with a recombinant DNA mixture of the aforementioned gene library in a conventional manner. Transformants of about 15000 strains were obtained as Cm resistant strains. These transformants were replicated on a minimum medium (5 g/L of glucose, 12.8 g/L of Na_2HPO_4, 3 g/L of KH_2PO_4, 0.5 g/L of NaCl, 1 g/L of NH_4C1, 40 μg/ml of L-threonine, 40 μg/ml of L-methionine) not containing arginine and uracil, and the minimum medium not containing L-arginine, but containing only 50 μg/ml of

uracil, and screened for a strain in which arginine auxotrophy and uracil auxotrophy were restored, or a strain in which arginine auxotrophy was restored. Strains in which arginine auxotrophy was restored recovered both of arginine auxotrophy and uracil auxotrophy. A plasmid harbored in one of such strains was designated as pl9, and the strain harboring it was designated as JEF8/pl9. The structure of pl9 is shown in Fig. 1.

The *Escherichia coli* JEF8/pl9 was designated as *Escherichia coli* AJ13574, and it was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry (postal code 305-8566, 1-3 Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan) on January 28, 1999, and received an accession number of FERM P-17180, and transferred from the original deposit to international deposit based on Budapest Treaty on January 6, 2000, and has been deposited as deposition number of FERM BP-6989.

<4> Acquisition of plasmid complementing arginine and uracil auxotrophy

A plasmid was prepared from JEF8/pl9, in a conventional manner, and used for re-transformation of the JEF8 strain. The obtained transformants could grow in the minimum culture medium not containing L-arginine and uracil, and its auxotrophy for both of L-arginine and uracil was restored. Therefore, it was found that the plasmid contained a gene complementing the auxotrophy for both of L-arginine and uracil caused by deletion of *car*B in the *Escherichia coli* strain.

Further, this plasmid was introduced into the *car*A mutant of *Escherichia coli,* RC50 (*carA50, tsx*$^-$*273*, λ^-, *rpsL135* (str^R), *malT1* (λR), *xylA7*, *thi*$^-$*1*; *Mol. Gen. Genet.*, 133, 299 (1974)). Since the strain introduced with the plasmid was able to grow in the minimum culture medium not containing arginine and uracil, the plasmid was also found to have a gene

complementing the auxotrophy for both of L-arginine and uracil caused by *carA* mutation of the *Escherichia coli* strain.

<5> Nucleotide sequence analysis of pl9

Among the DNA sequence of pl9, the nucleotide sequence of about 4.8 kb from the *Hind*III side of the multi-cloning site of the vector to the *Hind*III site contained in the insertion DNA fragment was determined. The nucleotide sequencing was performed by using Rohdamin Terminator Cycle Sequencing Kit (produced by ABI) according to the method of singer. The obtained nucleotide sequence is shown as SEQ ID NO: 1 in Sequence Listing. From analysis of a consensus sequence which located in the upstream region of this gene, it was estimated that two open reading frames (open reading frame from 283rd G to 1461st A and open reading frame from 1756th G to 4808th T) were contained in this sequence. The nucleotides of the 162nd (TGCATA) to 194th (TATAAT), the 185th (TGCATA} to 213rd (TAAACT), the 203rd (TTGAAT) 230th (TATCAA), or the 224th (TTATCA to 251st (TAAAAA) can be estimated to be a promoter region for regulating the transcription.

The amino acid sequences encoded by these open reading frames are represented with the nucleotide sequences. The amino acid sequences were also shown in SEQ ID NOS; 2 and 3. A protein database (GenBank CDS) was searched for sequences exhibiting homology with these amino acid sequences. As a result, it was found that the 5' open reading frame showed high homology (about 40%) with *carA* gene products of *Escherichia coli, Bacillus subtillis* and so forth, and the 3' open reading frame showed high homology with known *carB* gene products of *Escherichia coli, Bacillus stearothermophilus* and so forth (about 40 to 50%). Therefore, it was suggested that these open reading frames coded for *carA* and *carB*, respectively.

<6> Introduction of *carA* and *carB* into wild-type strain of coryneform bacteria

pl9 was introduced into the *Brevibacterimn flavum* wild strain 2247 (AJ14067) by the electric pulse method (Japanese Patent Laid-open NO. 2-207791). The transformants were selected as chloramphenicol resistant strains on a CM2G plate medium (containing 10 g of polypeptone, 10 g of yeast extract, 5 g of glucose, 5 g of NaCl, 15 g of agar in 1 L of pure water, pH 7.2) containing 5 μg/ml of chloramphenicol to obtain 2247/pl9.

Example 2: Production of L-arginine by coryneform bacteria introduced with *carA* and *carB*

<1> Preparation of shuttle vector

First, a plasmid vector autonomously replicable in both of *Escherichia coli* cells and coryneform bacterium cells was newly produced as a plasmid used for introducing the *carA* and *carB* genes into coryneform bacteria.

A vector containing a drug resistance gene of *Streptococcus faecalis* was constructed first. The kanamycin resistant gene of *Streptococcus faecalis* was amplified by PCR from a known plasmid containing that gene. The nucleotide sequence of the kanamycin resistant gene of *Streptococcus faecalis* has already been clarified (Trieu-Cuot, P. and Courvalin, P., *Gene,* 23(3), 331-341 (1983)). The primers shown as SEQ ID NOS: 4 and 5 were synthesized based on that sequence, and PCR was performed by using pDG783 (Anne-Marie Guerout-Fleury *et at., Gene,* 167, 335-337 (1995)) as a template to amplify a DNA fragment containing the kanamycin resistant gene and its promoter.

The obtained DNA fragment was purified by SUPRECO2 produced by the Takara Shuzo, then fully digested with restriction enzymes *Hind*III and *Hinc*II, and blunt-ended. The blunt-ending was attained by using Blunting Kit produced by Takara Shuzo. This DNA fragment was mixed with and ligated to a DNA fragment, which had been obtained by

performing PCR using the primers shown as SEQ ID NOS: 6 and 7 and pHSG399 (see S. Takeshita et al., *Gene, 6l*, 63-74 (1987)) as a template, purifying and blunt-ending the resulted amplification product. The ligation reaction was performed by DNA Ligation Kit ver. 2 produced by Takara Shuzo. Competent cells of *Escherichia coli* JM109 (produced by Takara Shuzo) were transformed with the ligated DNA, plated on L medium (10 g/L of Bacto-trypton, 5 g/L of Bacto-yeast extract, 5 g/L of NaCl, 15 g/L of agar, pH 7.2) containing 10 μg/ml of IPTGH (isopropyl-β-D-thiogalactopyranoside), 40 μg/ml of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and 25 μg/ml of kanamycin, and cultured overnight. The emerged blue colonies were picked up, and separated into single colonies to obtain transformant strains.

Plasmids were prepared from the transformant strains by the alkali method (Text for Bioengineering Experiments, Edited by the society for Bioscience and Bioengineering, Japan, p.105, Baifukan, 1992), and restriction maps were prepared. One having a restriction map equivalent to that of Fig. 2 was designated as pKl. This plasmid is stably retained in *Escherichia coli,* and imparts kanamycin resistance to a host. Moreover, since it contains the *lacZ'* gene, it is suitably used as a cloning vector.

The plasmid pAM330 extracted from *Brevibacterium lactofermentum* ATCC13869 (see Japanese Patent Laid-open No. 58-67699) was fully digested with a restriction enzyme *Hind*III, and blunt-ended. This fragment was ligated to a fragment obtained by fully digesting the aforementioned pK1 with a restriction enzyme *Bsa*AI. *Brevibacterium lactofermentum* ATCC13869 was transformed with the ligated DNA. The transformation was performed by the electric pulse method (see Japanese Patent Laidopen No. 2-207791). Transformants were selected on a M-CM2B plate (10 g/L of polypeptone, 10 g/L of yeast extract, 5 g/L of NaCl, 10 μg/L of biotin, 15 g/L of agar, pH 7.2) containing 25 μg/ml of kanamycin. After cultivation for 2 days, colonies were picked up, and separated into single colonies to obtain

the transfonmants. Plasmid DNA was prepared from the transformants, and restriction maps were prepared. One having the same restriction map as that of Fig. 3 was designated as pSFK6. This plasmid can autonomously replicate in both of *Escherichia coli* and coryneform bacteria, and imparts kanamycin resistance to a host.

2 Introduction of *car*A and *car*B genes into coryneform bacteria and production of L-arginine

The aforementioned pSFK6 wan digested with SmaI and *Hind*III. The product was ligated to *carA* and *carB* gene fragments, which had been obtained by digesting the plasmid p19 prepared from JEF8/pl9F in a conventional manner with a restriction enzyme *Xba*I, blunt-ending the product by using Blunting Kit produced by Takara Shuzo, and further digesting the product with a restriction enzyme *Hind*III, to obtain a plasmid pcarAB, which contained the carA and carB genes and could autonomously replicate in coryneform bacteria.

pcarAB was introduced into *Brevibacterium flavam* AJ11345 and AJ11336 by the electric pulse method (Japanese Patent Laid-open No. 2-207791). Transformants were selected on a M-CM2B plate (10 g/L of polypeptone, 10 g/L of yeast extract, 5 g/L of glucose, 5 g/L of NaCl, 15 g/L of agar, pH 7.2) containing 25 μg/ml of kanamycin as kanamycin resistant strains. As control, transformants were obtained by similarly introducing pSFK6 into AJ11345 and AJ11336.

Each of the aforementioned transformants was plated on an agar medium containing 0.5 g/dl of glucose, 1 g/dl of polypeptone, 1 g of yeast extract, 0.5 g/dl of NaCl and 5 μg/l of chloramphenicol, and cultured at 31.5°C for 20 hours. One inoculating loop of the obtained cells were inoculated to a medium containing 4 g/dl of glucose, 6.5 g/dl of ammonium sulfate, 0.1 g/dl of KH_2PO_4, 0.04 g/dl of $MgSO_4$, 0.001 g/dl of $FeSO_4$, 0.01 g/dl of $MnSO_4$, 5

μg/dl of VB_1, 5 μg/dl of biotin, 45 mg/dl of soybean hydrolysates (as an amount of N), and cultured in a flask at 31.5°C for 50 hours with shaking. The amounts of L-arginine produced by each strain were shown in Table 1.

The strains introduced with the *carA* and *carB* gene showed improved L-arginine productivity compared with the strains introduced only with the vector.

Table 1

Strain/plasmid	L-arginine (g/dl)
AJ11345/pSFR6	1.33
AJ11345/pcarAB	1.39
AJ11336/pSFK6	0.71
AJ11336/pcarAB	0.79

Incorporation by Reference

Each reference, patent application or patent publication cited by or referred to in this disclosure is incorporated by reference in its entirety. Any patent document to which this application claims priority is also incorporated by reference in its entirety. Specifically, priority document Japan 11-24149, filed February 1, 1999 is hereby incorporated by reference.

Modifications and other embodiments

Various modifications and variations of the described products and concept of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed is not intended to be limited to such specific embodiments. Various modifications of the described modes for carrying out the invention which are obvious to those skilled in the biological or chemical arts or related fields are intended to be within the scope of the following claims.